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Specific analogues uncouple transport, signalling, oligo-ubiquitination and endocytosis in the yeast Gap1 amino acid transceptor

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Summary

The *Saccharomyces cerevisiae* amino acid transceptor Gap1 functions as receptor for signalling to the PKA pathway and concomitantly undergoes substrate-induced oligo-ubiquitination and endocytosis. We have identified specific amino acids and analogues that uncouple to certain extent signalling, transport, oligo-ubiquitination and endocytosis. L-lysine, L-histidine and L-tryptophan are transported by Gap1 but do not trigger signalling. Unlike L-histidine, L-lysine triggers Gap1 oligo-ubiquitination without substantial induction of endocytosis. Two transported, non-metabolizable signalling agonists, β-alanine and D-histidine, are strong and weak inducers of Gap1 endocytosis, respectively, but both causing Gap1 oligo-ubiquitination. The non-signalling agonist, non-transported competitive inhibitor of Gap1 transport, L-Asp-γ-L-Phe, induces oligo-ubiquitination but no discernible endocytosis. The Km of L-citrulline transport is much lower than the threshold concentration for signalling and endocytosis. These results show that molecules can be transported without triggering signalling or substantial endocytosis, and that oligo-ubiquitination and endocytosis do not require signalling nor metabolism. Oligo-ubiquitination is required, but apparently not sufficient to trigger endocytosis. In addition, we demonstrate intracellular cross-induction of endocytosis of transport-defective Gap1^{Y395C} by ubiquitination- and endocytosis-deficient Gap1^{K9R,K16R}. Our results support the concept that different substrates bind to partially overlapping binding sites in the same general substrate-binding pocket of Gap1, triggering divergent conformations, resulting in different conformation-induced downstream processes.

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Introduction

In yeast and other cells, a common response to starvation for a specific nutrient is the induction of a high-affinity transporter for the uptake of trace amounts of substrate from the medium. Addition of ample substrate to such starved cells typically provokes endocytic internalization of the transporter followed by sorting to the multivesicular body (MVB) and degradation in the vacuole/lysosome (Magasanik and Kaiser, 2002; Lauwers *et al*., 2010). Ubiquitination is required for endocytosis, and addition of substrate generally induces a transient increase in oligo- and poly-ubiquitinated forms, which is generally detected as discrete increases in the apparent size of the transporter after separation by electrophoresis. The general amino acid permease Gap1 of *Saccharomyces cerevisiae* has been studied extensively as a model system for this type of substrate-induced transporter downregulation (Jauniaux and Grenson, 1990; Chen and Kaiser, 2002; Lauwers *et al*., 2010). The E3 ubiquitin ligase Rsp5 ubiquitinates Gap1 at the N-terminal lysines 9 and 16 (Soetens *et al*., 2001). Although oligo-ubiquitination was shown to be sufficient for endocytic internalization, K63 poly-ubiquitination by the concerted action of Rsp5 and the redundant proteins, Bul1,2, is needed for Gap1 vacuolar sorting through the MVB pathway (Lauwers *et al*., 2009; 2010). Similar observations on the pivotal role of ubiquitination in endocytosis have been made for mammalian nutrient transporters (Melikian, 2004; Zahniser and Sorkin, 2009).

Our work has revealed that at least some of the starvation-induced nutrient transporters, including Gap1 (Donaton *et al*., 2003), the Pho84 phosphate (Giots *et al*., 2003) and the Mep2 ammonium (Van Nuland *et al*., 2006) transporters, also function as receptors for rapid activation of the protein kinase A (PKA) pathway upon addition of their substrate. One of the best-characterized responses to

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nutrient activation of PKA is the rapid increase in trehalase activity, which is correlated with its phosphorylation on PKA consensus sites (Hirimburegama *et al.*, 1992; Schepers *et al.*, 2012). We proposed the name transceptors for proteins combining transporter and receptor functions (Holsbeeks *et al.*, 2004). Previous screening of substrate analogues has identified molecules that are not transported by the transceptor but can trigger transceptor-dependent signalling: e.g. L-Leu-Gly for Gap1 (Van Zeebroeck *et al.*, 2009) and glycerol-3-phosphate for Pho84 (Popova *et al.*, 2010). In addition, this previous work identified analogues acting as competitive inhibitors of transport but unable to trigger signalling: L-Asp-γ-L-Phe for Gap1 (Van Zeebroeck *et al.*, 2009) and phosphonoacetic acid for Pho84 (Popova *et al.*, 2010). This indicated that binding of a molecule into the substrate binding site is not enough to trigger signalling and that a signalling agonist must be able to induce a specific conformational change in the transceptor.

Several studies on substrate-induced endocytic internalization of transporters have focused on the relationship between transport of the substrate and induction of endocytosis. Gap1 mutant proteins, deficient in transport of basic amino acids or all amino acids, no longer undergo endocytosis after addition of these non-transported amino acids (Cain and Kaiser, 2011). Transport-defective mutant forms of the Fur4 uracil permease (Seron *et al.*, 1999) and the Ftr1 iron transporter (Felice *et al.*, 2005) in yeast and the uric acid/xanthine transporter, *An*UapA, in *Aspergillus nidulans* (Gournas *et al.*, 2010), failed to undergo internalization, which was taken as evidence that transport is required for triggering endocytosis. Previous work has shown that yeast cells contain two glucose sensors, Snf3 and Rgt2, and one amino acid sensor, Ssy1, in the plasma membrane that are structurally similar to regular transporters but have lost the capacity to transport their substrate (Forsberg and Ljungdahl, 2001). Hence, these transporter-like proteins are clearly able to recognize and respond to their former substrates without requirement for complete transport in order to trigger their signalling pathway. It is also not known whether a substrate can be transported through a carrier without provoking endocytosis or whether on the other hand transport through the carrier passageway necessarily triggers endocytosis (Kriel *et al.*, 2011). The discovery of the receptor function in some of the starvation-induced transporters has raised the question whether there is a mechanistic connection between the induction of signalling and endocytosis. In addition to substrate-induced endocytosis, several stress conditions, like heat shock, or use of protein synthesis inhibitors can also trigger endocytosis of these transporters (Seron *et al.*, 1999; Andre and Haguenauer-Tsapis, 2004; Lin *et al.*, 2008; Nikko and Pelham, 2009; Keener and Babst, 2013), although the effect of such conditions depends on the organism (Apostolaki *et al.*, 2009; Gournas *et al.*, 2010). The underlying mechanisms are not well understood.

In the present work, we have made use of specific chemical compounds, either amino acids or analogues, to investigate the connection between transport, metabolism, induction of endocytosis and oligo-ubiquitination, and induction of signalling in Gap1. We have discovered three transported amino acids that do not trigger signalling and found that one of these also does not trigger substantial endocytosis. This suggests that different substrates elicit different conformational changes when they move through the passageway of a transporter and shows that signalling and endocytosis are independently triggered. In addition, as previously demonstrated for signalling, we show that induction of endocytosis does not require metabolism but apparently needs elicitation of a specific conformational change in the transporter. We have also found that oligo-ubiquitination of Gap1 is triggered by compounds that do not trigger substantial endocytosis, indicating that an additional modification is required to initiate the endocytic internalization process. Our results support the concept that different substrates bind to partially overlapping binding sites in the same general substrate-binding pocket, that this triggers divergent conformations in the protein and therefore results in different conformation-induced downstream processes.

Results

Identification of transported non-signalling amino acids

We have previously reported amino acids and non-metabolized analogues that are transported by Gap1 and trigger its signalling function for activation of the PKA pathway, as inferred from activation of the trehalase enzyme (Donaton *et al.*, 2003; Van Zeebroeck *et al.*, 2009). Screening of all protein amino acids surprisingly revealed that L-histidine, L-lysine and L-tryptophan do not trigger signalling (Fig. 1A). Although Gap1 is well known as a broad-specificity permease, transporting all naturally occurring L-amino acids, we measured the initial uptake rate of these amino acids to make sure that they were well transported under our experimental conditions. Using radiolabelled amino acids, we found that transport of L-histidine, L-lysine and L-tryptophan in nitrogen-starved cells was mostly Gap1-dependent (Fig. 1B). These three non-signalling amino acids also did not sustain the start-up of growth as sole nitrogen source, with the exception of L-tryptophan, which supported slow growth after a long lag phase (Fig. 1C). L-Asparagine, a good nitrogen source used as control, sustained a fast start-up of growth. Only in the case of L-citrulline, growth was entirely dependent on Gap1 at the concentration tested.

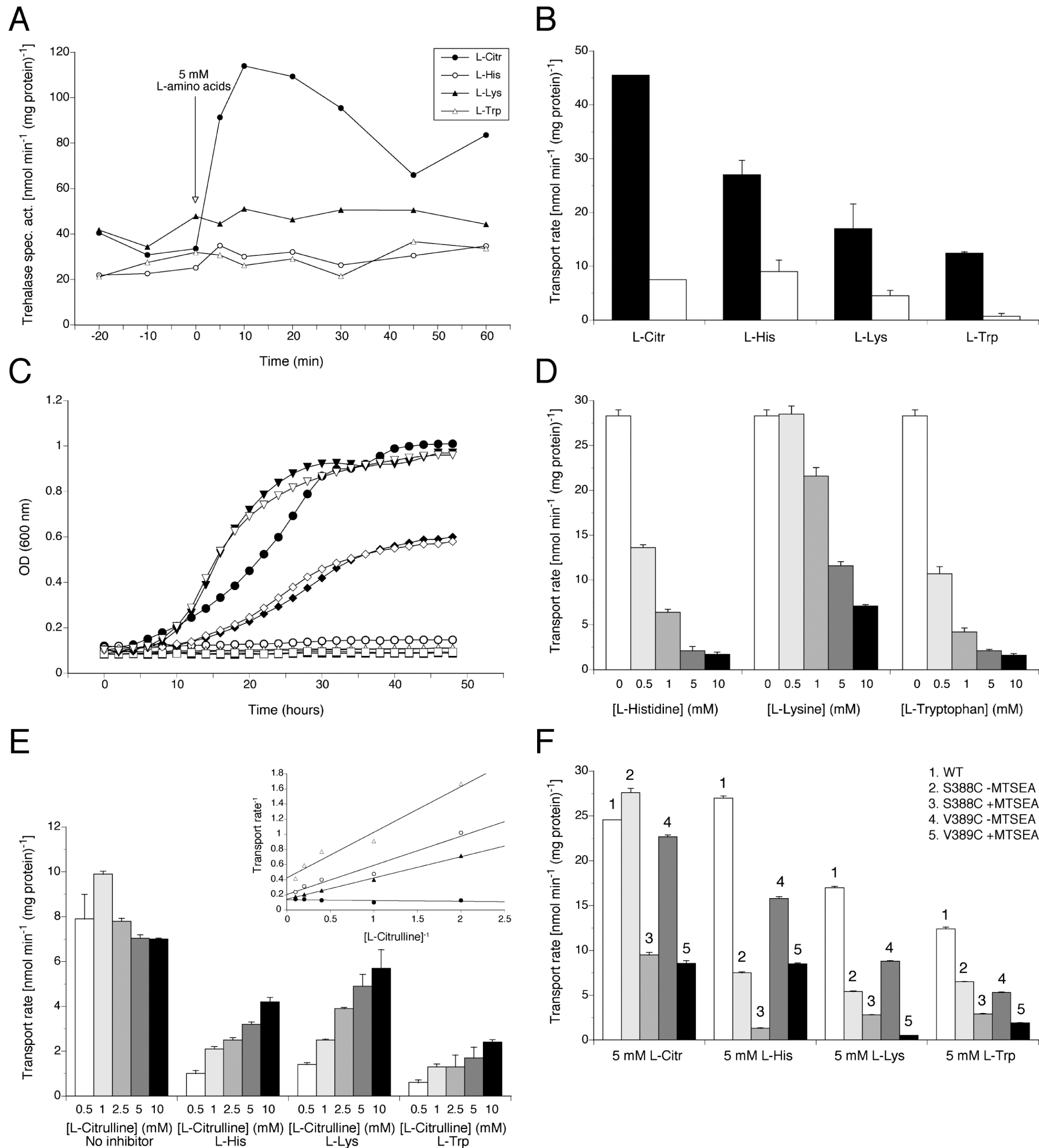

Fig. 1. Identification of transported, partially or largely competitive inhibitors without signalling capacity.
A. Activation of the PKA target trehalase in the wild-type strain after addition of 5 mM L-citrulline (●), L-histidine (○), L-lysine (▲) or L-tryptophan (△) to nitrogen-starved cells.
B. Gap1-dependent uptake. Transport of 5 mM L-citrulline, L-histidine, L-lysine or L-tryptophan in wild-type (black bars) and *gap1Δ* (white bars) strains.
C. The three non-signalling amino acids are very poor nitrogen sources. Growth on 5 mM L-citrulline (●, ○), L-histidine (▲, △), L-lysine (■, □), L-tryptophan (◆, ◊) or L-asparagine (▼, ▽) in wild-type (closed symbols) and *gap1Δ* (open symbols) strains.
D. L-histidine, L-lysine and L-tryptophan act as inhibitors of Gap1 transport. Transport of 1 mM L-citrulline measured in the presence of different concentrations L-histidine, L-lysine and L-tryptophan (0, 0.5, 1, 5 and 10 mM, white bars to black bars).
E. L-histidine, L-lysine and L-tryptophan act as partially or largely competitive inhibitors of Gap1 transport. Transport of five concentrations (0.5, 1, 2.5, 5 and 10 mM, white bars to black bars) of L-citrulline measured without inhibitor or in the presence of 0.125 mM L-histidine, 0.5 mM L-lysine or 0.125 mM L-tryptophan. These values are also shown as a Lineweaver-Burk plot (inset): no inhibitor (●), or 0.125 mM L-histidine (○), 0.5 mM L-lysine (▲), or 0.125 mM L-tryptophan (△).
F. Transport of the non-signalling amino acids is reduced by mutagenesis of Ser^{388} or Val^{389} to cysteine. Transport of 5 mM L-citrulline, L-histidine, L-lysine or L-tryptophan by a wild-type (1), $gap1^{S388C}$ (2, 3) and a $gap1^{V389C}$ (4, 5) strain, without (2, 4) or with (3, 5) pre-addition of 10 mM MTSEA.
Error bars in (A) to (F) represent standard deviation (s.d.) between biological repeats.

Non-signalling and signalling amino acids seem to bind through distinct interactions within a promiscuous binding pocket

The three non-signalling amino acids, L-histidine, L-lysine and L-tryptophan acted as inhibitors of L-citrulline uptake (Fig. 1D). In the case of L-lysine or L-histidine the inhibition was purely or largely competitive, respectively, while for L-tryptophan there was a clear non-competitive component (Fig. 1E). Based on Fig. 1E, the inhibition constants were determined as $K_i(His) = 0.0025$ mM, $K_i(Lys) = 0.0095$ mM and $K_i(Trp) = 0.0033$ mM. As mentioned above, tryptophan addition also resulted in an intermediate phenotype in terms of its ability to support growth (Fig. 1C). This indicates that these non-signalling amino acids apparently bind into the same binding pocket of Gap1 as the signalling amino acid, L-citrulline, but in a distinct way from the signalling substrate.

To obtain further evidence for this conclusion, we have made use of two residues, Ser^{388} and Val^{389}, which were previously found by Substituted Cysteine Accessibility Method (SCAM), and whose side-chains are exposed into the amino acid binding pocket of Gap1 (Van Zeebroeck *et al*., 2009). Covalent modification of the $Gap1^{S388C}$ or $Gap1^{V389C}$ proteins with the sulphydryl-reactive reagent MTSEA (2-aminoethyl methanethiosulphonate hydrobromide) blocked signalling by both transported and non-transported signalling agonists (Van Zeebroeck *et al*., 2009; Rubio-Texeira *et al*., 2012). Here we show that, in contrast to the signalling amino acids, transport of the non-signalling amino acids was already reduced in strains expressing the *gap1*^{S388C} or *gap1*^{V389C} allele before addition of MTSEA (Fig. 1F). This confirms the previous results suggesting that different substrates bind in a somewhat different way in the same promiscuous amino acid binding pocket. Addition of MTSEA, however, caused a further drop in the transport activity for all three non-signalling amino acids. Even though non-signalling and signalling amino acids interact with key elements of the substrate binding pocket, these results suggest that they have partially overlapping binding sites, which may result in partially different translocation trajectories through the transporter. As a result, slightly different conformational changes may be generated during the transport of the molecule. In support of this possibility, it has recently been shown that the *An*UapA substrate purine analogue, allopurinol, uses a partially different substrate translocation trajectory compared to the regular substrate, xanthine (Diallinas, 2013).

Non-signalling amino acids inhibit signalling by regular amino acids

Subsequently, we tested whether the three non-signalling amino acids are able to inhibit L-citrulline-induced Gap1 signalling (Fig. 2A–C). For that purpose, we measured rapid activation of the classical PKA target, trehalase. All three non-signalling amino acids inhibited L-citrulline induced trehalase activation. However, in the case of L-lysine, a very high concentration was required to inhibit activation of trehalase by 5 mM L-citrulline (Fig. 2B, Fig. S1). Also in the case of inhibition of L-citrulline transport, L-lysine was a poor inhibitor, although in that case the difference with the other two amino acids, L-histidine and L-tryptophan, was not so pronounced (Fig. 1D). Concentrations causing 50% inhibition of L-citrulline mediated signalling with L-histidine or L-tryptophan (e.g. 5 mM) were not sufficient to cause inhibition by L-lysine (Fig. S1). The inhibition of signalling was mixed to largely competitive for all three non-signalling amino acids since we were able to counteract at least partially the inhibition with increasing L-citrulline concentrations (Fig. 2D). These results indicate that the three non-signalling amino acids compete with L-citrulline for interaction with the binding site that triggers signalling, although they themselves are unable to trigger signalling through that site. This further supports that different substrates bind in different ways into the same binding pocket, that interaction with the amino acid binding site is not enough to trigger signalling, and that the amino acid apparently has to be able to induce an appropriate conformational change in Gap1 after binding to this site.

L-lysine triggers oligo-ubiquitination but not endocytosis of Gap1

We subsequently tested whether the transported non-signalling amino acids were able to trigger Gap1 endocytic internalization. Since L-tryptophan showed intermediate phenotypes for the start-up of growth, the inhibition of L-citrulline uptake and internalization of Gap1 (Fig. 1C and E and Fig. S2), we focused on L-histidine and L-lysine. In contrast to cells growing on poor nitrogen sources, such as urea or proline, nitrogen-starved cells typically show Gap1-GFP distribution between the plasma membrane and the vacuolar lumen (the latter derived from residual GFP after hydrolysis of Gap1-GFP). Addition of L-citrulline to nitrogen-starved cells triggers endocytosis and vacuolar sorting of Gap1-GFP (Fig. 3A). Similarly, addition of L-histidine also triggered internalization of Gap1-GFP. On the other hand, the membrane-localized Gap1-GFP signal remained unchanged after addition of L-lysine. This result suggests that L-lysine is unable to trigger substantial Gap1 endocytosis. Moreover, L-lysine was able to inhibit L-citrulline-induced endocytosis (Fig. 3B). Concentrations higher than 50 mM L-lysine were able to counteract internalization of Gap1 triggered by 5 mM L-citrulline. This competition assay also confirmed that L-lysine apparently interacts with the same binding site as L-citrulline. Remarkably, even at a concentration of 100 mM, L-lysine did not

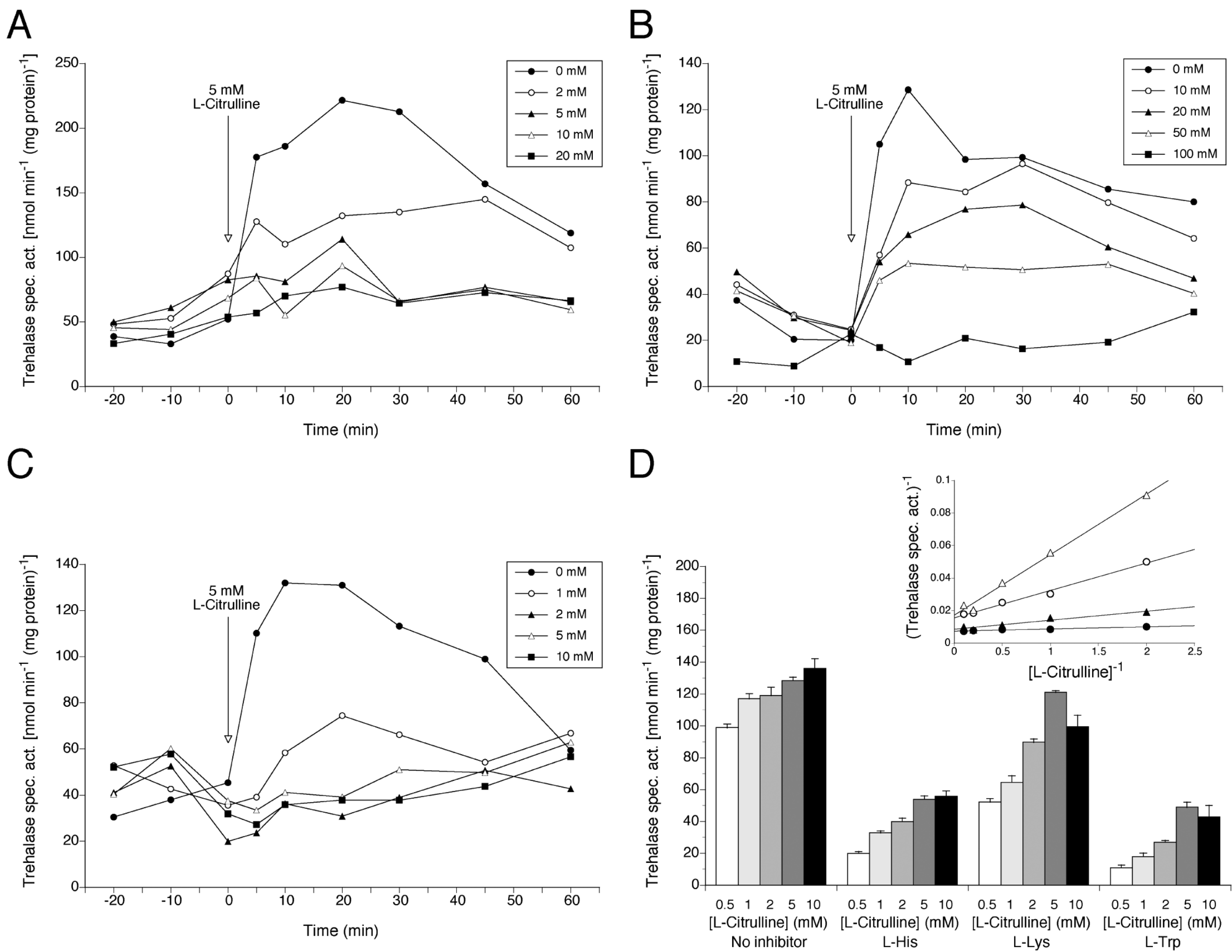

Fig. 2. All three non-signalling amino acids act as partially or largely competitive inhibitors of L-citrulline induced trehalase activation.
A–C. Activation of the PKA target trehalase in nitrogen-starved cells of the wild-type strain after addition of (A) 5 mM L-citrulline in the presence of 0 mM (●), 2 mM (○), 5 mM (▲), 10 mM (△) or 20 mM (■) L-histidine; (B) 2 mM L-citrulline in the presence of 0 mM (●), 10 mM (○), 20 mM (▲), 50 mM (△) or 100 mM (■) L-lysine; (C) 5 mM L-citrulline in the presence of 0 mM (●), 1 mM (○), 2 mM (▲), 5 mM (△) or 10 mM (■) L-tryptophan.
D. Activity of trehalase was measured 20 min after addition of the indicated L-citrulline concentrations in the absence or presence of 1 mM L-histidine, 10 mM L-lysine or 1 mM L-tryptophan. These values are also shown as a Lineweaver-Burk plot (inset): no inhibitor (●), 1 mM L-histidine (○), 10 mM L-lysine (▲) or 1 mM L- tryptophan (△). Error bars represent s.d. between biological repeats.

elicit substantial endocytosis of Gap1-GFP (Fig. 3B). This is, to the best of our knowledge, the first identified substrate that does not trigger internalization of its permease after accumulation of the latter has been induced by starvation for its substrate. We also noticed that L-lysine caused conspicuous enlargement of the vacuole, which is known to be a storage place for basic amino acids (Shimazu *et al.*, 2005).

Gap1 has been reported to show high affinity for L-histidine, L-lysine and L-tryptophan (30, 9–13 and 3 μM respectively) (Grenson *et al.*, 1970). This raises the question whether there might be a relationship between the higher substrate affinity and the reduced ability to trigger signalling or endocytosis of Gap1. L-arginine also has a high affinity for Gap1 (8 μM) (Grenson *et al.*, 1970), thus we decided to test the effect of this amino acid on Gap1 signalling and endocytosis. In contrast to the three other high-affinity substrates, exposure to either 1 or 5 mM L-arginine triggered trehalase activation to the same extent as L-citrulline at the same concentrations (Figs S3A and S4A). Moreover L-arginine also triggered rapid endocytosis (Fig. S3B). Hence, we conclude that higher substrate affinity is not necessarily associated with a reduced ability to trigger signalling or endocytosis of Gap1.

The use of mM concentrations of amino acids for our signalling studies stems from the fact that these concentrations always provide us with reproducible results for trehalase activation, our PKA-activation read-out,

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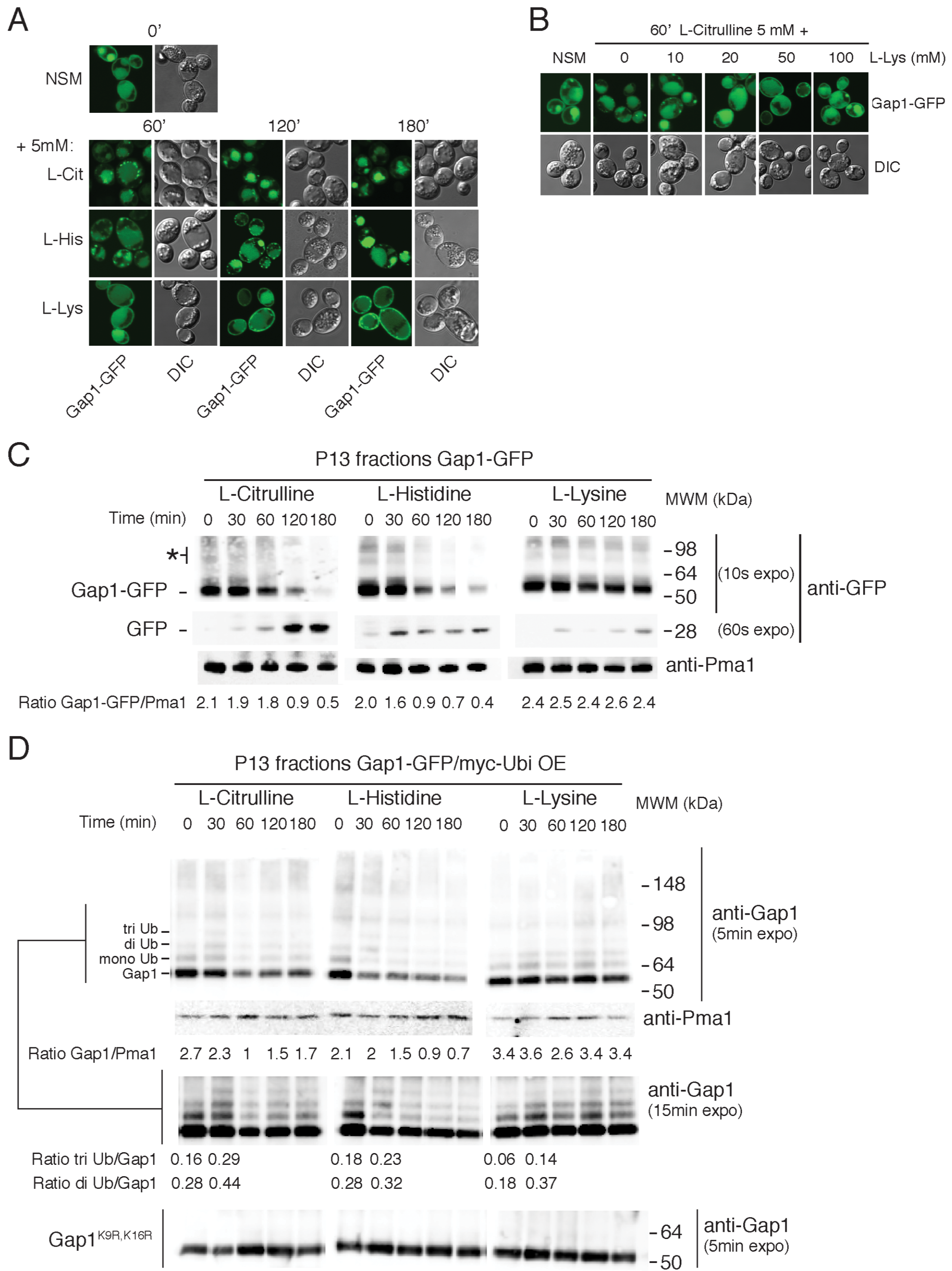

(Donaton *et al.*, 2003). Moreover, concentrations of L-citrulline in the range of 1–5 mM are nearly exclusively taken up by Gap1, which provides specificity for Gap1-mediated signalling (Donaton *et al.*, 2003). Since concentrations in this range are much above the Gap1 Km values for these substrates, we wondered whether using lower concentrations in the μM range would allow us to observe similar differences in signalling and endocytosis. However,

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Fig. 3. The transported non-signalling amino acid L-lysine does not trigger substantial endocytosis but triggers Gap1 oligo-ubiquitination, and counteracts L-citrulline induced internalization.
A. Gap1-GFP localization in wild-type cells is shown 60, 120 and 180 min after addition of 5 mM of L-citrulline or the non-signalling amino acids L-histidine or L-lysine, to nitrogen-starved cells (nitrogen starvation medium, NSM).
B. Gap1-GFP localization in wild-type cells is shown before and 60 min after addition of 5 mM L-citrulline, either without (+0 mM L-lysine), or together with different concentrations of L-lysine (10, 20, 50 or 100 mM) to nitrogen-starved cells.
C. Analysis of Gap1-GFP stability in membrane-enriched (P13) fractions at different time points (0, 30, 60, 120 and 180 min) after addition of L-citrulline, L-histidine or L-lysine to nitrogen-starved cells. Western blot was carried out with HRP-anti-GFP antibody, showing levels of Gap1-GFP (10 s exposure), or free GFP at 60 s of exposure of the same blot. Normalization of the loading is shown with anti-Pma1 antibody. Luminescent arbitrary units (LAU) $\times 10^{-6}$ are shown as ratio between the Gap1-GFP band and Pma1 band for each time point.
D. Analysis of Gap1 ubiquitination status in nitrogen-starved cells expressing endogenous *GAP1* and induced with 10 μM $CuSO_4$ for 30 min prior to addition of nitrogen source, for moderate overexpression (OE) of *myc*-ubiquitin from the P_{CUP1}-*myc*-Ubi URA3 plasmid, pMRT7. P13 fractions were collected at different time points (0, 30, 60, 120 and 180 min) after addition of 5 mM L-citrulline, L-histidine or L-lysine to nitrogen-starved cells. Upper panels: Western blot with anti-Gap1 antibody. Bottom panels: Western blot with anti-Pma1 antibody as loading control. Luminescent arbitrary units (LAU) $\times 10^{-6}$ are shown as ratio between the Gap1 band and Pma1 band for each time point to assess relative disappearance of the Gap1 band, consistent with endocytosis. The ratios between di- or tri-ubiquitinated to non-ubiquitinated Gap1 are also shown to assess the relative increase of the former with respect to the latter after addition of each nitrogen source. A Western blot from cells expressing the non-ubiquitinatable $Gap1^{K9R,K16R}$ subjected to identical treatment is also shown as control to confirm that upper bands observed above the Gap1 band in the wild-type blots are ubiquitinated forms of the transceptor.

when the concentration of L-citrulline was reduced to below 500 μM, both trehalase activation and endocytosis were absent (Fig. S4A and B). Hence, the threshold concentration for both signalling and endocytosis appears to be much higher than the Km for transport. This result supports the conclusions from the experiments with L-lysine that transport by itself is not sufficient to trigger signalling or endocytosis. Strong levels of endocytosis were only fully achieved at concentrations above 1 mM (Fig. S4B), confirming that the concentrations near 5 mM of amino acid agonist are optimal to study both Gap1-mediated signalling and endocytosis. Furthermore, mM concentrations did not present any problems in terms of causing toxicity as cells did not show abnormal morphologies or cell lysis under the microscope and they were perfectly able to grow in the presence of a 5 mM concentration of L-citrulline (Fig. 1C).

In parallel with the analysis of Gap1-GFP internalization, we took samples for analysis of the stability and ubiquitination status of Gap1. Cells were collected before and after addition of the amino acid to nitrogen-starved cells, extracts were prepared and samples of membrane-enriched (P13) protein fractions were analysed for the level of Gap1-GFP by Western blot (Fig. 3C). A weak signal of free GFP was sometimes detected before addition of the nitrogen compound, reflecting the Gap1-GFP fraction already sorted to the vacuole in the nitrogen-starved cells. Addition of L-citrulline or L-histidine to nitrogen-starved cells led to decreased stability of Gap1-GFP and simultaneous increase in free GFP at the later time points after addition of the amino acid, indicative of endocytosis and vacuolar degradation. On the other hand, incubation for up to 3 h in the presence of L-lysine did not substantially change the levels of Gap1-GFP recovered in fractions from equal time points, and free GFP was only very weakly accumulated. Intensity of the Gap1-GFP signal as luminescent arbitrary units (LAU) was compared in the same Western blots to that of Pma1, used as loading control. The ratio of Gap1-GFP to Pma1 was clearly reduced for time points after 30 min in the case of L-citrulline and L-histidine but not L-lysine (Fig. 3C).

Transporter oligo-ubiquitination preceding its endocytosis has been difficult to detect because of weak antibody binding and because it only appears as a transient phenomenon due to the ensuing breakdown of the transporter. To discern the appearance of oligo-ubiquitinated species after addition of each amino acid more clearly, we expressed the plasmid pP_{CUP1}-*myc*-*UBI* (pMRT7; Rubio-Texeira and Kaiser, 2006) in a wild-type strain containing the endogenous *GAP1* gene. Cells were incubated as above for collection of P13 fractions before and different times after addition of the amino acid, with the only exception that 30 min before addition of the amino acid, 10 μM of $CuSO_4$ was added to mildly induce expression of *myc*-ubiquitin (Ub) from the plasmid [full promoter expression would be achieved by 100 μM of $CuSO_4$ (Helliwell *et al.*, 2001)]. In this case, levels of Gap1 species were monitored by Western blot using Gap1-specific antibody. Gap1 forms were also quantitatively measured through LAU determination. Identification of anti-Gap1 immunoreactive 60–90 kDa forms as nitrogen-source induced oligo-ubiquitinated forms of Gap1 was verified in two ways. First, mere induction of myc-Ub did not enhance appearance of di- and tri-ubiquitinated bands (Fig. S5A). Only the mono-ubiquitin band was consistently observed from time zero on, possibly related to the background levels of Gap1 being sorted to the vacuole in nitrogen-starved cells. Second, we have performed the same experiment with a strain coexpressing $CuSO_4$ inducible *myc*-Ubi and $Gap1^{K9R,K16R}$. This mutant form of Gap1 lacks the two main lysine ubiquitin acceptors K9 and K16, and consequently cannot be endocytosed upon addition of nitrogen compounds to nitrogen-starved cells (Fig. 3D and Fig. S5B–D) (Soetens *et al.*, 2001). In fractions taken from this strain no 60–90 kDa immunoreactive forms were accumulated above the size

corresponding to non-ubiquitinated Gap1. Ratio of the sizes consistent with di- and tri-ubiquitinated Gap1 compared to non-ubiquitinated Gap1 in the wild-type indicated an increase of the former within a period of 30 min after addition of the amino acid (Fig. 3D). This indicated that although L-lysine did not induce substantial endocytosis, it still triggered a similar but more permanent oligo-ubiquitination as the other amino acids that trigger endocytosis (L-citrulline and L-histidine). Quantification revealed a two- to threefold increase, similar to the intensity of the transient increase in oligo-ubiquitination observed with L-citrulline. An increase in oligo-ubiquitination, therefore, seemed by itself insufficient to efficiently trigger Gap1 endocytosis under our experimental conditions.

Interestingly, in these Western blot experiments, a mild background of anti-Gap1 immunoreactive, high-molecular-weight forms (≥ 98 kDa) was consistently observed before and after addition of the different nitrogen compounds (Fig. 3C and D). In order to discern whether these bands corresponded to highly poly-ubiquitinated species, we analysed P13 fractions from cells expressing Gap1^{K9R,K16R}-GFP. Unexpectedly, samples taken from these cells exposed to 5 mM L-citrulline still showed the high-molecular-weight forms in Western blots probed with antibodies against GFP (Fig. S5C). This was not due to an artefact of the GFP tag since similar results were also obtained for the strain coexpressing Gap1^{K9R,K16R} and *myc*-Ubi (Fig. S5D). These forms accumulated even more strongly in the Western blots from Gap1^{K9R,K16R}-GFP or Gap1^{K9R,K16R} (Fig. S5C and D), compared to blots of wild-type Gap1 (Fig. 3C and D). This suggests either that these Gap1 forms result from ubiquitination on alternative acceptor sites (this seems rather unlikely since in such case we would expect to observe also oligo-ubiquitinated forms), or that instead, they represent aggregated forms of Gap1 with itself or with yet unidentified proteins. Since Gap1 is a protein known to enter rafts (Lauwers and Andre, 2006; Lauwers *et al*., 2007), it is also possible that these high-molecular-weight bands result from detergent-resistant aggregates of Gap1 with lipids. In any case, our results consistently indicated transient changes in the oligo-ubiquitinated species of Gap1 (sizes ranging from 60 to 90 kDa) regardless of whether the nitrogen compound was able to trigger substantial endocytosis.

Non-metabolizable, transported and signalling amino acid analogues trigger different levels of oligo-ubiquitination and endocytosis

The two non-metabolizable amino acid analogues, β-alanine and D-histidine, are transported by Gap1 and are able to trigger Gap1-dependent PKA signalling (Donaton *et al*., 2003) (Fig. S6A). Moreover they are acting largely as competitive inhibitors of L-citrulline transport (Fig. S6B and C). When these two analogues were tested for their ability to induce endocytosis of Gap1-GFP in nitrogen-starved cells, fluorescence microscopy showed that β-alanine, but not D-histidine, induced rapid internalization of Gap1-GFP, similar to the control L-asparagine (Fig. 4A). This result shows that amino acid-induced endocytosis of Gap1 can be triggered in the absence of further metabolism of the transported substrate.

Consistent with this observation, immunoblots of P13 fractions taken from the wild-type strain expressing *myc*-Ubi as shown for Fig. 3, showed increased levels of di- and tri-ubiquitinated forms of Gap1 with respect to non-ubiquitinated Gap1 30 min after addition of each of the three amino acid analogues, including D-histidine (Fig. 4B). This indicated that even though oligo-ubiquitination is triggered in the presence of D-histidine, this event is not sufficient to trigger complete internalization of Gap1. That these bands corresponded to ubiquitinated forms of Gap1 was again confirmed by their absence in Western blots of the strain coexpressing Gap1^{K9R,K16R} and *myc*-Ubi subjected to the same treatment (Fig. 4B, bottom panel). The result with D-histidine demonstrates that transport through Gap1 can occur without triggering substantial endocytosis and therefore confirms the previous results obtained with L-lysine. Since, in contrast to L-lysine, D-histidine triggers signalling, this result also shows that signalling to the PKA pathway is not necessarily associated with simultaneous induction of endocytosis. Interestingly, a single change from the L- to the D-form of the same amino acid reverses its ability to cause signalling and endocytosis. The most logical explanation for this observation is that the two forms elicit different conformational changes in the transceptor after binding and/or during their translocation.

L-Asp-γ-L-Phe triggers oligo-ubiquitination but not endocytosis

L-Asp-γ-L-Phe is a non-signalling competitive inhibitor of Gap1 amino acid transport (Van Zeebroeck *et al*., 2009). Because of its nature as competitive inhibitor we were interested in testing its potential effect on Gap1 ubiquitination and endocytosis. Although we initially confirmed the absence of short-term uptake of this dipeptide (Van Zeebroeck *et al*., 2009), we observed a very slow Gap1-independent uptake of the dipeptide, in contrast to L-citrulline, over a period of 3 h after its addition to nitrogen-starved cells (Fig. 5A). In order to test its effect on ubiquitination and endocytosis we first wanted to analyse whether this long-term uptake of the dipeptide occurs through peptide transporters and whether it is metabolized, in which case it could affect Gap1 ubiquitination and endocytosis via changes in the intracellular amino acid pool once it is transported inside the cells (Chen and Kaiser,

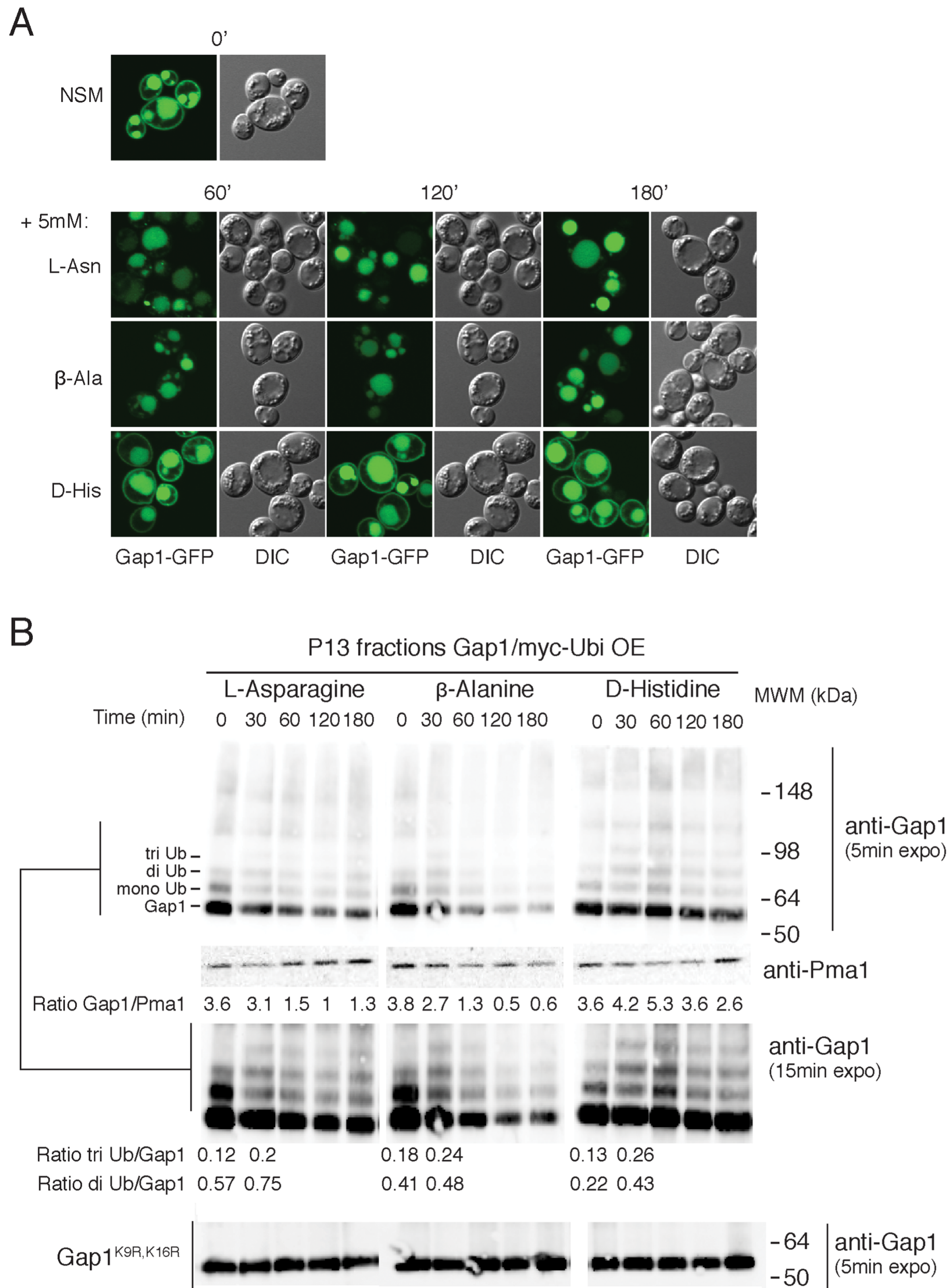

Fig. 4. Non-metabolizable, transported and signalling amino acid analogues cause different effects for oligo-ubiquitination and endocytosis.
A. Gap1-GFP localization in wild-type cells is shown 60, 120 and 180 min after addition of 5 mM of either the regular transported and signalling amino acid L-asparagine or the non-metabolizable, transported and signalling amino acid analogues β-alanine or D-histidine to nitrogen-starved cells.
B. Analysis of Gap1 ubiquitination status in nitrogen-starved cells expressing endogenous *GAP1* and induced with 10 μM $CuSO_4$ for 30 min prior to addition of nitrogen source, for expression of *myc*-ubiquitin from the P_{CUP1}-*myc*-Ubi URA3 plasmid, pMRT7. P13 fractions were collected at different time points (0, 30, 60, 120 and 180 min) after addition of 5 mM L-asparagine, β-alanine or D-histidine to nitrogen-starved cells. Upper panels: Western blot with anti-Gap1 antibody. Bottom panels: Western blot with anti-Pma1 antibody as loading control. Luminescent arbitrary units (LAU) $\times 10^{-6}$ are shown as ratio between the Gap1 band and Pma1 band for each time point to assess relative disappearance of the Gap1 band, consistent with endocytosis. The ratios between di- or tri-ubiquitinated to non-ubiquitinated Gap1 are also shown to assess the relative increase of the former with respect to the latter after addition of each nitrogen source. A Western blot from cells expressing the non-ubiquitinatable $Gap1^{K9R,K16R}$ subjected to identical treatment is also shown as control to confirm that upper bands observed above the Gap1 band in the wild-type blots are ubiquitinated forms of the transceptor.

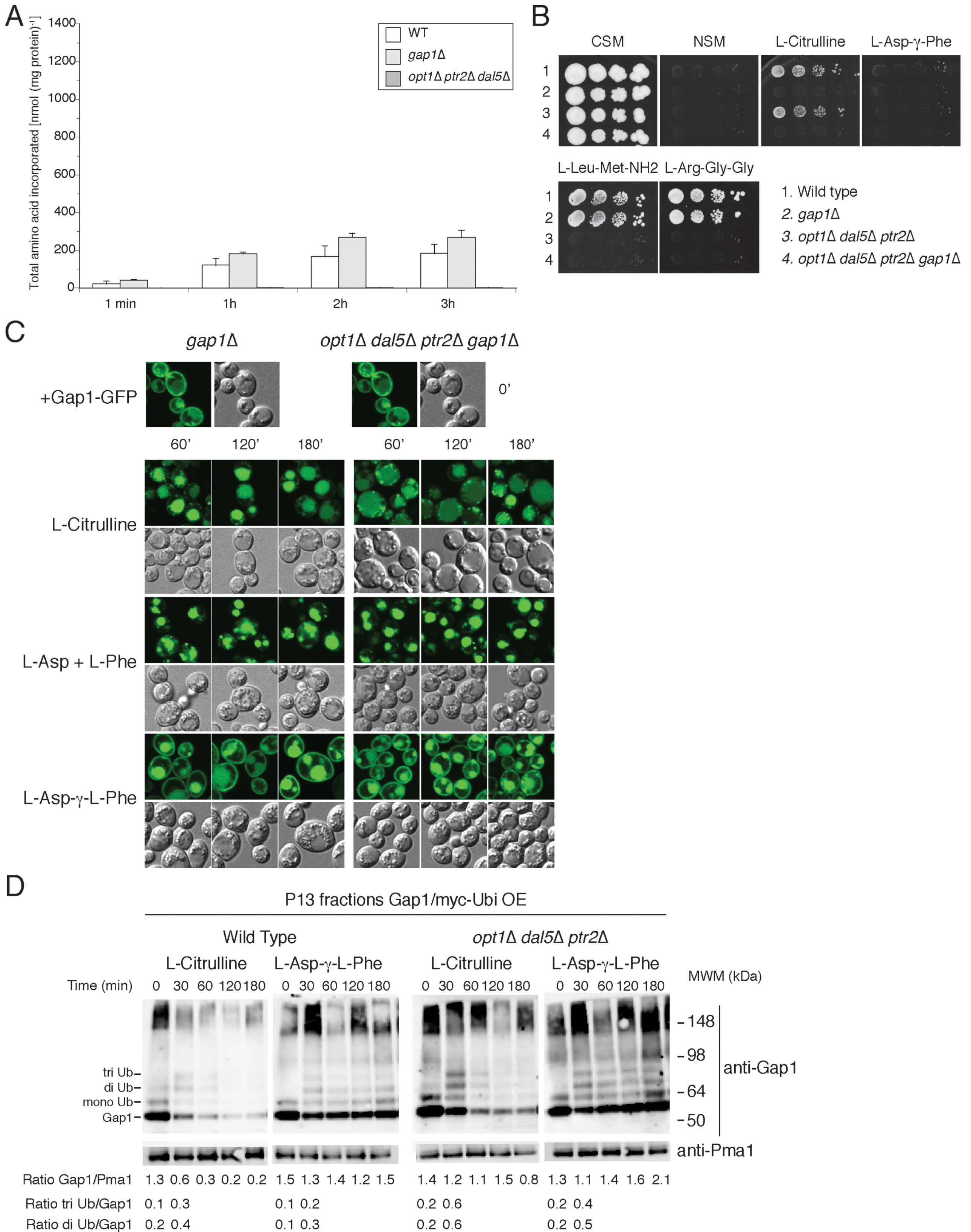
A
Total amino acid incorporated [nmol (mg protein)-1]
1400
1200
1000
800
600
400
200
0
1 min
1h
2h
3h
WT
gap1Δ
opt1Δ ptr2Δ dal5Δ
B
CSM
NSM
L-Citrulline
L-Asp-γ-Phe
L-Leu-Met-NH2
L-Arg-Gly-Gly
1. Wild type
2. gap1Δ
3. opt1Δ dal5Δ ptr2Δ
4. opt1Δ dal5Δ ptr2Δ gap1Δ
C
gap1Δ
opt1Δ dal5Δ ptr2Δ gap1Δ
+Gap1-GFP
0'
60'
120'
180'
L-Citrulline
L-Asp + L-Phe
L-Asp-γ-L-Phe
D
P13 fractions Gap1/myc-Ubi OE
Wild Type
opt1Δ dal5Δ ptr2Δ
L-Citrulline
L-Asp-γ-L-Phe
Time (min)
0 30 60 120 180
MWM (kDa)
148
98
64
50
anti-Gap1
tri Ub
di Ub
mono Ub
Gap1
anti-Pma1
Ratio Gap1/Pma1 1.3 0.6 0.3 0.2 0.2 1.5 1.3 1.4 1.2 1.5 1.4 1.2 1.1 1.5 0.8 1.3 1.1 1.4 1.6 2.1
Ratio tri Ub/Gap1 0.1 0.3 0.1 0.2 0.2 0.6 0.2 0.4
Ratio di Ub/Gap1 0.2 0.4 0.1 0.3 0.2 0.6 0.2 0.5

Fig. 5. The non-transported and non-signalling competitive inhibitor of Gap1-mediated transport, L-Asp-γ-L-Phe, cannot trigger endocytosis but triggers ubiquitination in the wild-type strain.
A. Progressive intracellular accumulation of radioactively labelled L-Asp-γ-L-Phe after addition of 5 mM of this compound to nitrogen-starved cells. Strains: wild-type (black bars), *gap1*Δ (white bars) and *opt1*Δ *dal5*Δ *ptr2*Δ (grey bars). Error bars represent s.d. between biological repeats.
B. Growth of 1/10 serial dilution spottings of nitrogen pre-starved cells of the strains wild-type, *gap1*Δ, *opt1*Δ *dal5*Δ *ptr2*Δ and *opt1*Δ *dal5*Δ *ptr2*Δ *gap1*Δ on plates of nitrogen starvation medium (NSM) without or supplemented with 1 mM of L-citrulline, or L-Asp-γ-L-Phe. The same cells spotted in complete supplemented medium (CSM) are shown as positive growth control. Growth of the same cells in NSM + 1 mM of the dipeptide Leu-Met-NH2 or the tripeptide L-Arg-Gly-Gly is shown as control of peptide use as nitrogen source due to peptide carrier uptake.
C. Localization of wild-type Gap1-GFP expressed in the strains *gap1*Δ and *opt1*Δ *dal5*Δ *ptr2*Δ *gap1*Δ is shown before and 60, 120 and 180 min after addition of 5 mM L-Asp-γ-L-Phe. The same cells exposed to 2.5 mM L-aspartate plus 2.5 mM L-phenylalanine is shown as control that the dipeptide constituent amino acids are able to induce endocytosis.
D. Analysis of Gap1 ubiquitination status in nitrogen-starved cells expressing endogenous *GAP1* (from the wild-type or the triple deletion mutant *opt1*Δ *dal5*Δ *ptr2*Δ) and induced with 10 μM $CuSO_4$ for 30 min prior to addition of nitrogen source, for expression of *myc*-ubiquitin from the P_{CUP1}-*myc*-Ubi URA3 plasmid, pMRT7. P13 fractions were collected at different time points (0, 30, 60, 120 and 180 min) after addition of 5 mM L-Asp-γ-L-Phe to nitrogen-starved cells. Upper panels: Western blot with anti-Gap1 antibody. Bottom panels: Western blot with anti-Pma1 antibody as loading control. Luminescent arbitrary units (LAU) $\times 10^{-6}$ are shown as ratio between the Gap1 band and Pma1 band for each time point to assess the relative disappearance of the Gap1 band, consistent with endocytosis. The ratios between di- or tri-ubiquitinated to non-ubiquitinated Gap1 are also shown to assess the relative increase of the former with respect to the latter after addition of each nitrogen source.

2002; Merhi and Andrè, 2012). Transport was completely abolished by deletion of the three major peptide carriers present in *S. cerevisiae*, i.e. in the mutant strain *opt1*Δ *dal5*Δ *ptr2*Δ (Fig. 5A) (Hauser *et al*., 2000; 2001; Cai *et al*., 2007). However, L-citrulline transport was still inhibited by L-Asp-γ-L-Phe in this triple mutant, indicating interaction of the dipeptide with Gap1 regardless of the absence of peptide carrier-mediated transport (Fig. S7A and B). Growth on various dipeptides and tripeptides as only nitrogen source was impaired in cells deleted for these three major peptide carriers. For example, wild-type and *gap1*Δ cells could use 1 mM of Leu-Met-NH2 or L-Arg-Gly-Gly [two non-competitive inhibitors of Gap1-dependent L-citrulline transport (Van Zeebroeck *et al*., 2009)], indicating that these two peptides do not enter cells through Gap1 (Fig. 5B). However, the strain *opt1*Δ *dal5*Δ *ptr2*Δ could no longer use them as only N source, presumably because of its inability to take them up (Fig. 5B). In contrast, L-Asp-γ-L-Phe could not be used as only nitrogen source either by the wild-type or by the *gap1*Δ strain indicating that even when it is transported inside the cells it is not metabolized (Fig. 5A and B).

L-Asp-γ-L-Phe was therefore a good candidate to test ubiquitination and endocytosis by a non-transported substrate analogue, since it still inhibits L-citrulline transport in the *opt1*Δ *dal5*Δ *ptr2*Δ strain (Fig. S7) (Van Zeebroeck *et al*., 2009). Regardless of its uptake by the peptide carriers, this dipeptide was unable to induce endocytosis of Gap1-GFP, as shown in either wild-type or *opt1*Δ *dal5*Δ *ptr2*Δ strains (Fig. 5C). Thus, its interaction with Gap1 is not sufficient to cause Gap1 endocytosis. However, when we tested appearance of oligo-ubiquitinated forms in cells of the wild-type or the *opt1*Δ *dal5*Δ *ptr2*Δ strain expressing *myc*-Ubi upon exposure to L-Asp-γ-L-Phe, we clearly detected appearance and accumulation of di- and tri-ubiquitinated forms of Gap1 in both cases (Fig. 5D). Their accumulation was much more permanent than in the case of L-citrulline. Quantification revealed a two- to threefold increase, similar to the intensity of the transient increase in oligo-ubiquitination observed with L-citrulline. This indicated that although the interaction of L-Asp-γ-L-Phe with Gap1 does not suffice to cause Gap1 endocytosis it still causes substantial accumulation of oligo-ubiquitinated Gap1. This is to the best of our knowledge the first case of a non-transported molecule causing ubiquitination of a transporter (or transceptor). Moreover, this result confirms that oligo-ubiquitination is not sufficient *per se* to trigger endocytosis of a transporter (or transceptor), suggesting that additional changes e.g. in conformation or in post-translational modification may be needed to initiate endocytosis.

An alternative possibility for all the cases where we have observed an apparent lack of endocytosis is that endocytosis is masked by enhanced accumulation of newly synthesized Gap1 arriving at the plasma membrane. To evaluate this possibility we tested plasma membrane localization of Gap1-GFP after addition of the compounds that are unable to trigger substantial endocytosis, L-Lys, L-Asp-γ-L-Phe, and D-His, in conditions in which protein translation is abolished by addition of 50 μg ml^{-1} of the protein synthesis inhibitor, cycloheximide (Fig. S8). To ensure that translation was stopped at the beginning of the experiment, the cells were pre-incubated for 20 min in the presence of cycloheximide. If the stable plasma membrane signal results from accumulation of newly synthesized transceptor arriving to the plasma membrane, then the presence of cycloheximide should result in a similar disappearance of Gap1-GFP from the plasma membrane after addition of any of these compounds, as we observe with regular amino acids such as L-citrulline (Fig. S8). However, while L-citrulline caused clear endocytosis of Gap1-GFP even in the presence of

cycloheximide, the plasma membrane-localized Gap1-GFP signal remained unchanged for cells exposed for an equal period of time to the same concentration of L-Lys, L-Asp-γ-L-Phe, or D-His. This excludes that the maintenance of plasma membrane Gap1-GFP signal after addition of these compounds is due to secretion of newly synthesized protein, and supports that it is caused by the absence of efficient endocytosis. Investigation of the effect of these analogues for longer periods of time in the presence of cycloheximide was not possible due to the fact that exposure to cycloheximide longer than 1 h by itself causes endocytosis of many plasma membrane proteins, including Gap1 (Nikko and Pelham, 2009; MacGurn *et al*., 2011) (Fig. S8).

Non-signalling L-amino acids induced ubiquitination but no endocytosis of the poorly transporting mutant, Gap1^{Y395C}

We previously showed that the Gap1^{Y395C} protein, mutated in a residue located in TMDVIII has strongly reduced transport and signalling with regular amino acids (Van Zeebroeck *et al*., 2009). Transport of L-histidine and L-lysine was also strongly reduced in this mutant (Fig. 6A). When L-citrulline, L-histidine and L-lysine were added to nitrogen-starved cells we did not observe substantial disappearance of Gap1^{Y395C}-GFP from the plasma membrane (Fig. 6B). A similar lack of enhanced internalization was also observed when Gap1^{Y395C}-GFP was exposed to L-asparagine or to the non-metabolizable analogues β-alanine or D-histidine (Fig. S9). Even though the three non-signalling L-amino acids were unable to trigger endocytosis of this mutant form of Gap1, they still elicited oligo-ubiquitination of the mutated transceptor, as observed by detection of newly appearing di- and tri-ubiquitinated Gap1 (Fig. 6C). This further indicates that oligo-ubiquitination of Gap1 is not sufficient to elicit normal rates of endocytosis and that regular rates of transport are not required to trigger oligo-ubiquitination.

Wild-type Gap1 cross-triggers endocytosis of defective Gap1^{Y395C}

We have shown that the Gap1^{Y395C} protein is largely defective in transport and endocytosis with L-citrulline, L-histidine or L-lysine (Van Zeebroeck *et al*., 2009) (Fig. 6A and B). This raised the question whether wild-type Gap1 would be able to cross-trigger endocytosis of the defective Gap1^{Y395C} protein and, if so, whether this would depend on endocytosis of the wild-type Gap1 and/or its signalling activity. To investigate this issue, we constructed strains expressing genomic C-terminal mRFP-tagged wild-type Gap1 or ubiquitination/endocytosis deficient Gap1^{K9R,K16R}. After confirmation that the tagging did not affect transport of L-citrulline, L-histidine or L-lysine, we transformed the strains with a centromeric plasmid expressing C-terminal GFP-tagged wild-type Gap1 or Gap1^{Y395C} (Fig. S10A–C). Transport of L-citrulline, L-histidine and L-lysine took place in all these strains.

Next, we monitored localization of the mRFP- and GFP-tagged forms of Gap1 expressed in the same cells upon addition of L-citrulline, L-histidine and L-lysine to nitrogen-starved cells (Fig. 7). Addition of L-citrulline to cells expressing Gap1-mRFP and Gap1-GFP triggered endocytosis of both proteins. Interestingly, addition of L-citrulline to cells expressing Gap1-mRFP also triggered endocytosis of Gap1^{Y395C}-GFP expressed in the same cells (Fig. 7A and B). This indicates that L-citrulline can trigger endocytosis of Gap1^{Y395C}-GFP through its effect on Gap1-mRFP. This was also observed in the strain expressing Gap1^{K9R,K16R}-mRFP, which remains localized at the plasma membrane in all conditions (Fig. 7A and B). Hence, the effect is independent of simultaneous endocytosis of wild-type Gap1-mRFP, i.e. it excludes that endocytosis of Gap1^{Y395C}-GFP is due to association with Gap1-mRFP or to recruitment in the same endosomes as Gap1-mRFP. The addition of L-histidine also triggered endocytosis of Gap1^{Y395C}-GFP both in the strains expressing Gap1-mRFP and in the strains expressing Gap1^{K9R,K16R}-mRFP (Fig. 7C), indicating that Gap1 signalling to the PKA pathway is not involved in triggering cross-endocytosis.

L-lysine did not cause substantial endocytosis of Gap1-GFP or Gap1^{Y395C}-GFP expressed in a *gap1*Δ strain (Figs 3A and B and 6B) and this was also true in a strain expressing Gap1-mRFP (Fig. 7D). This indicates that L-lysine is unable to trigger the same cross-endocytosis that can be triggered by interaction of L-citrulline and L-histidine with wild-type Gap1-mRFP. On the other hand, L-lysine triggered endocytosis of both wild-type and Gap1^{Y395C}-GFP in a strain expressing Gap1^{K9R,K16R}-mRFP (Fig. 7D). This suggests that L-lysine may interact differently with Gap1^{K9R,K16R}-mRFP compared to wild-type Gap1-mRFP, or that the higher level of Gap1^{K9R,K16R} in the plasma membrane may strengthen the signalling that triggers endocytosis, resulting in the same cross-endocytosis as observed with L-citrulline and L-histidine. Overall, these results again indicate that transport of the substrate through a transceptor is not required to trigger its endocytosis.

Discussion

Transport does not always trigger PKA signalling

We have identified three amino acids, L-histidine, L-lysine and L-tryptophan, that are readily transported by Gap1, but do not trigger signalling to the PKA pathway. Partially competitive inhibition of L-citrulline transport and signalling

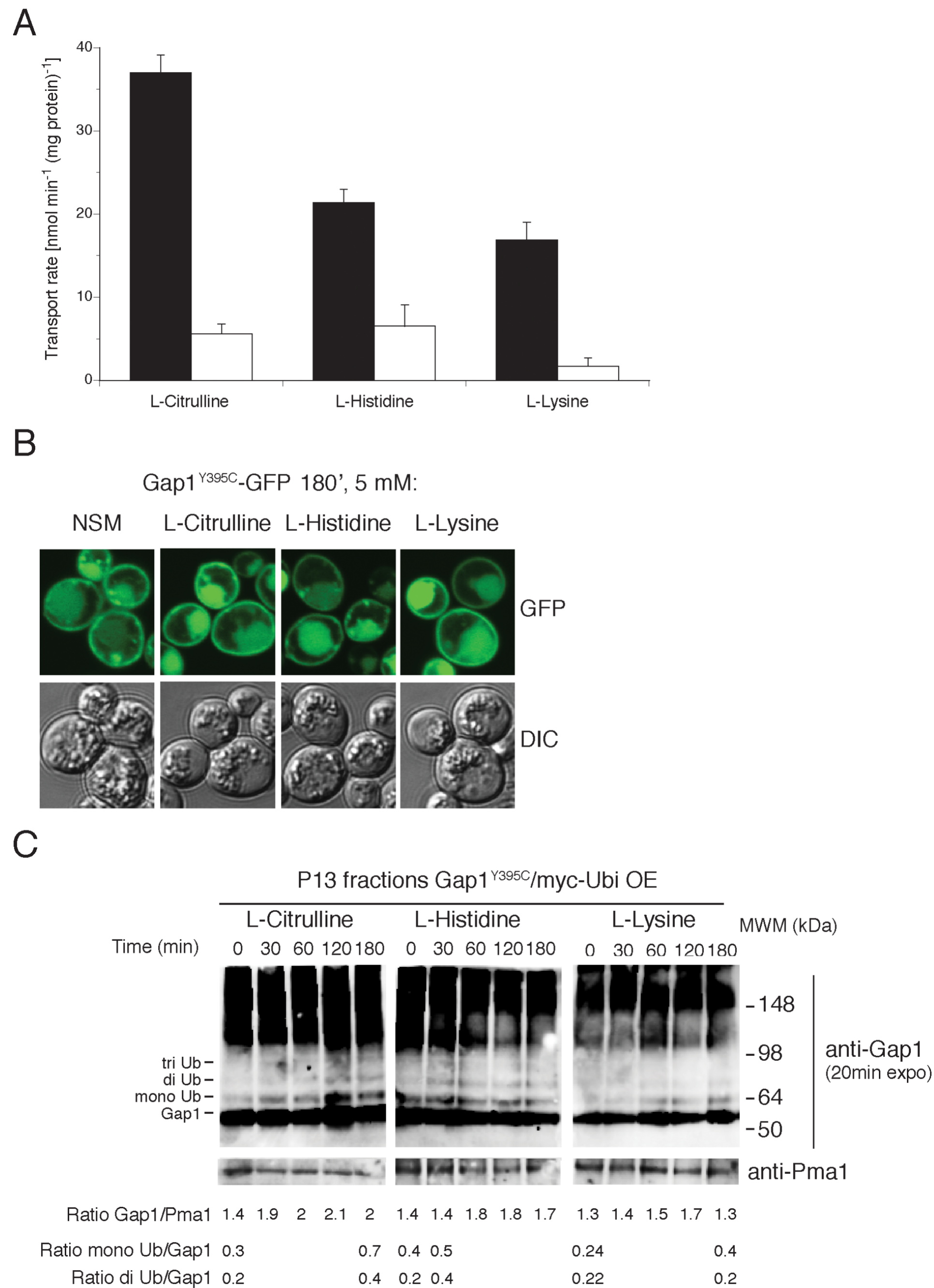

Fig. 6. Behaviour of nearly transport-inactive Gap1^{Y395C} in the presence of non-signalling amino acids L-histidine and L-lysine.
A. Transport of 5 mM L-citrulline, L-histidine or L-lysine in wild-type (black bars) or *gap1*^{Y395C} (white bars) strains. Error bars represent s.d. between biological repeats.
B. Gap1^{Y395C}-GFP localization is shown 0, 60, 120 and 180 min after addition of 5 mM L-citrulline, L-histidine or L-lysine to nitrogen-starved cells.
C. Analysis of Gap1 ubiquitination status in nitrogen-starved *gap1Δ* cells expressing Gap1^{Y395C} (from YCpGap1^{Y395C}, URA3 plasmid) and induced with 10 μM $CuSO_4$ for 30 min prior to addition of nitrogen source, for expression of *myc*-ubiquitin from the P_{CUP1}-*myc*-Ubi HIS3 plasmid, pMRT39. P13 fractions were collected at different time points (0, 30, 60, 120 and 180 min) after addition of 5 mM L-citrulline, L-histidine or L-lysine to nitrogen-starved cells. Upper panels: Western blot with anti-Gap1 antibody. Bottom panels: Western blot with anti-Pma1 antibody as loading control. Luminescent arbitrary units (LAU) × 10^{-6} are shown as ratio between the Gap1 band and Pma1 band for each time point to assess the relative disappearance of the Gap1 band, consistent with endocytosis. The ratios between di- or tri-ubiquitinated to non-ubiquitinated Gap1 are also shown to assess the relative increase of the former with respect to the latter after addition of each nitrogen source.

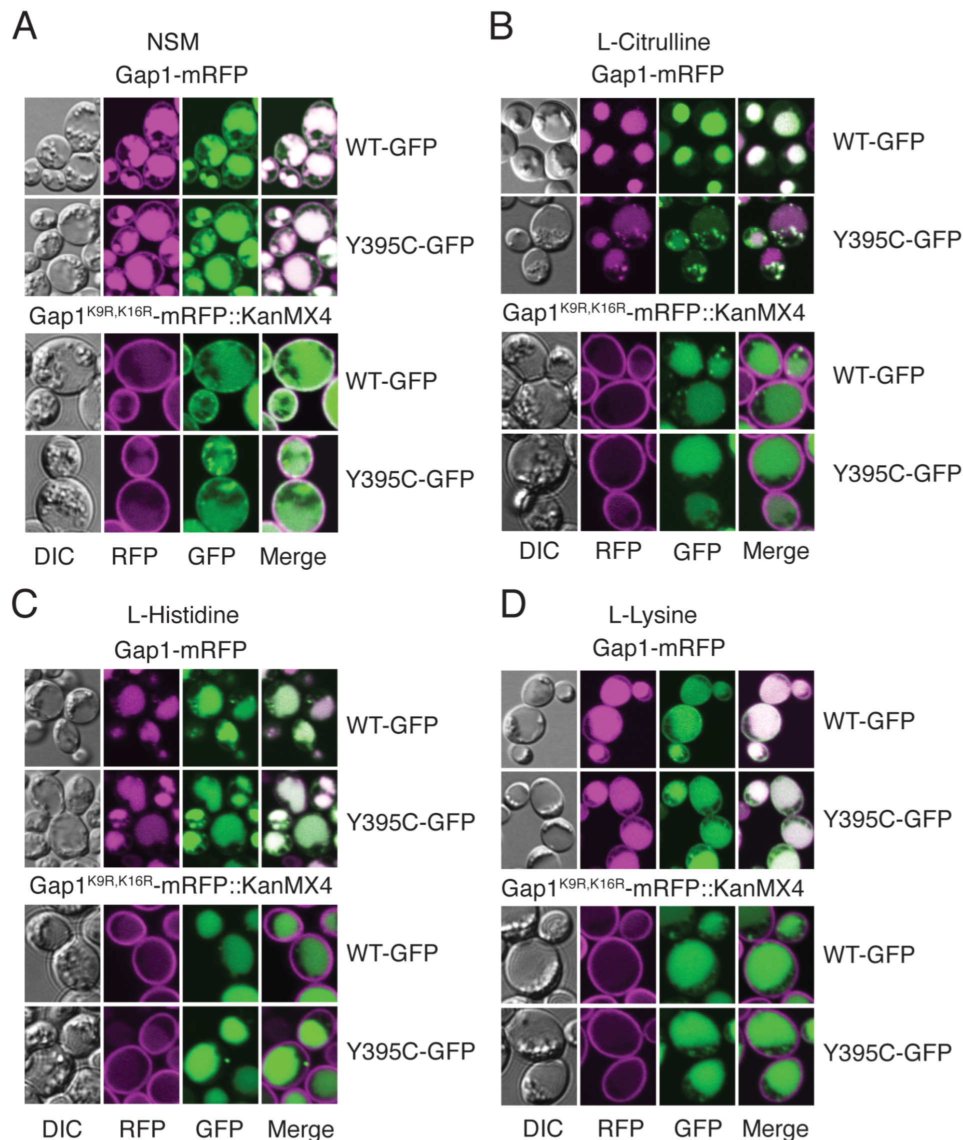

Fig. 7. Gap1 transport activity at the plasma membrane causes signalling- and endocytosis-independent cross-endocytosis of transport-defective Gap1. Nitrogen-starved cells of strains coexpressing genomic mRFP-tagged wild-type or Gap1^{K9R,K16R}, combined with plasmid-expressed GFP-tagged wild-type or nearly inactive Gap1 (Y395C), were monitored (A) for mRFP and GFP localization at 0 (NSM) and 60 min after addition of 5 mM (B) L-citrulline, (C) L-histidine or (D) L-lysine.

as well as SCAM analysis, indicate that they interact with a partially overlapping binding site as the regular amino acids, excluding that their inability to signal is due to binding to a completely different part of the transceptor. Their failure to trigger signalling, suggests that different substrates cause different conformational changes during transport through a permease and that these three amino acids do not elicit the conformational change required to trigger signalling. All three are also very poor nitrogen sources for yeast. Although this may suggest that the quality of the nitrogen source is relayed by Gap1 to the PKA pathway, the latter is contradicted by previous results indicating that certain non-metabolizable nitrogen sources, like β-alanine and D-amino acids, also trigger PKA signalling (Donaton *et al.*, 2003). Hence, whether the absence of Gap1 signalling by L-histidine, L-lysine and L-tryptophan has a physiological meaning, remains unclear. The conclusion that transport can take place without triggering signalling was further supported by the finding that L-citrulline concentrations below 500 μM were unable to trigger signalling in spite of the fact that the Km for L-citrulline uptake by Gap1 is only 37 μM (Van Zeebroeck *et al.*, 2009).

Substrate-induced transceptor endocytosis is not always coupled to substrate transport or signalling

Several studies have addressed the relationship between substrate transport and substrate-induced transporter endocytosis in yeast and other organisms such as *A. nidulans*. In these cases, generation of transport-defective permeases by mutagenesis was always accompanied by loss of substrate-induced endocytosis (Liu and Culotta, 1999; Seron *et al*., 1999; Felice *et al*., 2005; Jensen *et al*., 2009; Gournas *et al*., 2010). Recently, transport-defective mutants of Gap1 were also described in which loss of transport caused loss of endocytosis (Cain and Kaiser, 2011). In a separate work, a close correlation between transport inactivation and the rate of substrate influx in Sul2, a yeast sulphate transporter, was taken as evidence for 'use-dependent inactivation' (Jennings and Cui, 2012). In *A. nidulans*, a compound, 3-methylxanthine, was found for the uric acid/xanthine transporter *An*UapA which binds to the transporter without triggering endocytosis (Gournas *et al*., 2010). In this case, evidence was shown that mere binding of the high-affinity competitive ligand/inhibitor was not sufficient to cause endocytosis. Even though the *An*UapA N409D mutant held a Km value similar to the wild-type, no transport or endocytosis could be observed. All these results have led to the general view that transport of the substrate through the transporter is coupled to endocytosis. Our results here, demonstrate that L-Asp-γ-L-Phe, in spite of being a non-transported competitive inhibitor of Gap1 transport (Van Zeebroeck *et al*., 2009), also does not trigger endocytosis, mimicking the effect of 3-methylxanthine on *An*UapA. Identification of such compounds supports that mere binding of a molecule to the substrate binding site of the transporter (or transceptor) is not enough to trigger endocytosis (or signalling). Apparently, the molecule has to be able to induce a specific conformational change in the protein that enables either or both phenomena.

Examination of the non-signalling amino acids, L-histidine and L-lysine, for induction of endocytosis showed that, even though both are transported by Gap1, only L-histidine triggered endocytosis. Moreover, as for signalling, L-citrulline concentrations below 500 μM were unable to trigger endocytosis in spite of the fact that the Km for L-citrulline uptake by Gap1 is only 37 μM (Van Zeebroeck *et al*., 2009). These results contradict a direct mechanistic connection between signalling and the induction of endocytosis and argue against substrate transport always leading to endocytosis of the transporter/transceptor. Moreover, two other transported, non-metabolizable signalling agonists, β-alanine and D-histidine, also showed a differential ability to trigger endocytosis, the former being effective while the latter being largely ineffective. This further argues against a direct mechanistic connection between transport and endocytosis and shows that endocytosis does not require further metabolism of the transported nitrogen compound. D-histidine is the first non-metabolizable molecule discovered that triggers signalling without triggering endocytosis of a transceptor. The molecules L-histidine and D-histidine uncouple signalling from endocytosis in opposite ways. L-histidine does not trigger signalling but triggers endocytosis, while the opposite is true for D-histidine. This clearly shows that signalling and the induction of endocytosis are independent events triggered by the Gap1 transceptor. These results similarly demonstrate that substrate transport not always leads to endocytosis and also show that endocytosis does not require further metabolism of the transported nitrogen compound. The latter is consistent with previous work showing that non-metabolizable amino acids can trigger Gap1 endocytosis (Chen and Kaiser, 2002). These results and the ones presented here are consistent with differential properties of the substrates to cause conformational changes which form part of the transport cycle, not all of them leading to endocytosis, regardless of their transport rate and further intracellular metabolism.

Oligo-ubiquitination is apparently not enough to trigger endocytosis

Another unexpected outcome of this work is the observation that a non-transported ligand, L-Asp-γ-L-Phe, and transported substrates of Gap1, like L-lysine or D-histidine, are able to trigger different degrees of oligo-ubiquitination without triggering substantial endocytosis. This challenges the prevailing view in the literature that (oligo-) ubiquitination is sufficient to trigger endocytosis (Gitan and Eide, 2000; Shih *et al*., 2000; Hicke and Dunn, 2003; Horak, 2003; Dupre *et al*., 2004; Eguez *et al*., 2004; Liu *et al*., 2007; Nikko *et al*., 2008; Lauwers *et al*., 2010; Barberon *et al*., 2011). We are aware that detection of substrate-induced transporter oligo-ubiquitination is technically not straightforward. However, our conclusions are based on several independent and consistent results. First, we have observed permanent oligo-ubiquitination with L-lysine, D-histidine and L-Asp-γ-L-Phe for the wild-type Gap1 protein. Second, we also observed permanent oligo-ubiquitination with L-citrulline for the mutant Gap1Y395C protein. The increases are between two- and threefold, but the transient oligo-ubiquitination of Gap1 with a regular amino acid is also only between two- and threefold. Hence, the generally accepted phenomenon of Gap1 oligo-ubiquitination has the same intensity as the novel observation of oligo-ubiquitination without ensuing endocytosis. The transient versus more permanent character of the oligo-ubiquitination also fits well with the presence or absence of Gap1 endocytosis as followed independently

by GFP fluorescence microscopy. Hence, we feel confident that our observations genuinely demonstrate Gap1 oligo-ubiquitination without endocytosis.

Our results are different from those presented for the yeast copper transporter Ctr1, which was still ubiquitinated after mutagenesis of two main ubiquitination acceptor lysines located at the C-terminus, even though endocytosis was abolished. In that case it was indicated that ubiquitination on other residues was incapable of mediating copper-induced endocytosis (Liu *et al.*, 2007). However, in the cases we show here the oligo-ubiquitination observed is clearly K9 and K16-dependent, as it disappears in the corresponding mutant, Gap1^{K9R,K16R}. In addition, the oligo-ubiquitination triggered by, for example, D-histidine, is strikingly similar to that caused by the endocytosis-inducing amino acids such as L-citrulline or L-asparagine, excluding intracellular amino acid metabolism as the trigger.

Particularly interesting was the fact that the non-signalling competitive inhibitor of Gap1 transport, L-Asp-γ-L-Phe, was still able to cause Gap1 oligo-ubiquitination, in spite of, first, not being transported by Gap1 nor by other peptide carriers in the *opt1*Δ *dal5*Δ *ptr2*Δ strain; second, not being metabolized in either case and, third, not being able to trigger Gap1 endocytosis. Since this effect cannot be attributed to either direct or indirect transport of the dipeptide nor metabolism inside the cells, the only possible explanation is that its interaction with Gap1 causes a particular conformation in which the transceptor has the ability to interact with the Rsp5/Bul ubiquitin ligase complex. Since L-Asp-γ-L-Phe does not trigger internalization of Gap1 by endocytosis, this apparently results in a continuously increasing level of ubiquitinated Gap1 in the plasma membrane. This result clearly shows that oligo-ubiquitination *per se* is not sufficient to trigger endocytosis of a transceptor. The effect of the competitive inhibitor L-Asp-γ-L-Phe on Gap1 is reminiscent of the effect of the competitive inhibitor tryptophan on the LeuT amino acid transporter, which traps the transporter in an Open-to-Out conformation (Singh *et al.*, 2008). Similarly, progressive accumulation of oligo-ubiquitinated signal could result from L-Asp-γ-L-Phe locking Gap1 in a specific conformation susceptible to oligo-ubiquitination but not to endocytosis. In any case, our results highlight that particular substrates, even non-transported ones, elicit different levels of oligo-ubiquitination, most likely related to different conformations induced in Gap1, which might in turn result in alternative subsequent modifications and/or protein–protein interactions. Also in G-protein coupled receptors there is great variation in the requirement and the role of ubiquitination in endocytosis, indicating that additional modifications and/or conformational changes can trigger or may be required for endocytosis (Hislop and von Zastrow, 2011).

Cross-endocytosis of inactive Gap1 by active Gap1

Although the molecular mechanisms of substrate-induced endocytosis in nutrient transporters have been studied in great detail, there are still important unsolved questions. Gournas *et al.* (2010) have demonstrated that an active transporter can trigger endocytosis *in trans* of an inactive transporter even when the active transporter itself cannot be endocytosed. We now show that this is also the case for the Gap1 transceptor and that it occurs independently of its signalling function to the PKA pathway. Interestingly, this observation along with our observation on the existence of SDS-resistant, high-molecular-weight anti-Gap1-immunoreactive proteins present in Western blots from membrane enriched-fractions regardless of the ubiquitination status (still visible in blots of Gap1^{K9R,K16R}-containing extracts), may point to the possibility of this transporter undergoing homo- or hetero-oligomerization prior to endocytosis. In our experimental conditions, we used 2 h of wet transfer from polyacrylamide gel onto nitrocellulose membrane, as opposed to the usual time of 1 h used in most wet transfer experiments. Our longer incubation time, allowing for better accumulation of high-molecular-weight proteins in the blot membranes, may explain why these forms have not been regularly detected in previous Gap1 Western blots performed by other laboratories. The possibility of these being detergent-resistant oligomers of Gap1 either with itself or with other proteins is supported by other examples in the literature. It has, for example, recently been shown that the SUT1 protein from *Solanum tuberosum* forms homodimeric complexes associated with lipid raft-like microdomains in yeast as well as in plants and this association to microdomains is thought to affect its endocytosis and recycling (Krugel *et al.*, 2012). Mep transporters are also thought to oligomerize since coexpression of Mep3 with Mep1 or the inactive form Mep1$^{G412–413D}$ only restores *mep1–3* null mutant growth on ammonia in the first but not the latter case (Marini *et al.*, 2000). As mentioned in the introduction, Gap1 is also known to interact with sphingolipids and associate with lipid rafts (Lauwers *et al.*, 2007), so the question remains whether it does so as an oligomer rather than as a monomer. Oligomerization would be consistent with our trans-endocytosis and Western blot results and certainly deserves future investigation.

Gap1 trans-endocytosis strongly suggests that functional transporters activate or recruit a component that recognizes all Gap1 transporters, whether active or not. Recent results by Merhi and Andrè (2012) may provide an explanation in this respect. They showed that the arrestin-like Bul proteins are regulated by phosphorylation in an Npr1-dependent manner and bound to the 14-3-3 proteins in conditions that protect Gap1 against downregulation. In their work, induction of Gap1 endocytosis was triggered

through a heterologous system, i.e. by addition of ammonium, which is transported through its own Mep1–3 carriers. Ammonium transport and also its incorporation into glutamate, was required for release of the Bul proteins and Gap1 endocytosis. On the other hand, in substrate-induced endocytosis of Gap1, release of the Bul proteins through downregulation of Npr1, may be triggered by a signal originating from the active Gap1 transceptor itself. Subsequent binding of the Bul proteins to any Gap1 molecule, whether actively transporting or not, could then explain the cross-endocytosis observed in our work. The compounds discovered in this work that can uncouple signalling, transport, oligo-ubiquitination and endocytosis, represent powerful new tools to elucidate the molecular mechanisms involved in substrate-induced endocytosis of Gap1.

Overlapping binding sites and conformation-induced downstream processes

Gap1 is a very promiscuous transporter that apparently accepts many substrates and non-transported analogues into its main amino acid binding site. Following binding, conformational changes are generated that guide the transported substrates through the translocation trajectory to be delivered to the cytosol at the other side of the membrane. Non-transported analogues may follow part of this trajectory. The conformational changes occurring as a result of substrate/analogue binding and transport through the carrier are thought to trigger downstream processes like endocytosis and signalling. Other such processes may well exist as shown by the discovery of substrate-induced reversible attenuation of Gap1 transport activity (Risinger *et al.*, 2006). Our work now strongly suggests that different substrates and analogues do not bind in exactly the same way into the general amino acid binding pocket of the transporter, but rather have overlapping binding sites within this general pocket, and probably also do not follow exactly the same trajectory through the transporter, confer allopurinol and xanthine in the *Aspergillus* UapA transporter (Diallinas, 2013), or at least do not interact with the same amino acid residues along the trajectory. As a result, different substrates and analogues can trigger different conformations or can cause shorter- or longer-lasting durations of the same conformations. This may then in turn result in uncoupling of the different downstream processes that are observed as occurring simultaneously with a regular amino acid: signalling, transport, oligo-ubiquitination and endocytosis. Our results show that they can all be uncoupled to an unexpectedly large extent, and maybe with other substrates or analogues even complete uncoupling of all of these processes might be possible. The results also underscore the importance of conformational changes in transporters for triggering downstream processes, in agreement with previous studies on substrate-induced endocytosis (Gournas *et al.*, 2010). This highlights the similarity between the behaviour of transporters and receptors, providing further evidence that receptors may have evolved from transporters and that transceptors may represent an evolutionary transition between the two systems (Kriel *et al.*, 2011).

Conclusions

Our results support the concept that different transporter substrates and non-transported ligands bind to partially overlapping binding sites in the same general substrate-binding pocket of a transporter, triggering divergent conformations, resulting in different conformation-induced downstream processes. We have been able to uncouple the presumed link between transport and endocytosis, as accomplished also for transport and signalling. We have presented conditions in which transport does not trigger endocytosis and in which non-metabolizable amino acid analogues trigger endocytosis, indicating that the latter does not necessarily require metabolism of the transported substrate. In addition, we have shown that oligo-ubiquitination can be triggered independently of transport and without subsequent induction of substantial endocytosis. The non-transported and non-metabolizable inducers of oligo-ubiquitination and/or endocytosis as well as the demonstration of cross-endocytosis between transporting and transport-deficient forms of Gap1, provide convenient tools for future elucidation of the initial steps of recruitment and/or activation of the endocytic machinery by the Gap1 transceptor.

Experimental procedures

Strains and growth media

The *S. cerevisiae* strains used in this work are all isogenic to wild-type strain Σ1278b (Supplementary Table S1). All plasmids used are listed in Supplementary Table S2. For regular transport and trehalase experiments, the strain 21.983c (*gap1*Δ *ura3-52*) transformed with pFL38 (empty *URA3 CEN* plasmid), or YCpGAP1 carrying wild-type, S388C, V389C, or Y395C versions of the *GAP1* gene was employed as described previously (Van Zeebroeck *et al.*, 2009). For microscopy, the Gap1-sGFP tagged *CEN*-URA3 plasmid versions described in Rubio-Texeira *et al.* (2012) were employed. The plasmid pGAP1^{K9R,K16R}-sGFP was made by transfer of the Bsu36I–BspEI from pGAP1^{K9R,K16R} (Soetens *et al.*, 2001) into the pGAP1-sGFP (Rubio-Texeira *et al.*, 2012). For Western blot analysis of ubiquitinated species of Gap1, the strains were transformed with the *URA3*, 2 μ plasmid pMRT7 (pP$_{CUP1}$-*myc-UBI*; Rubio-Texeira and Kaiser, 2006) or the *HIS3*, 2 μ plasmid pMRT39. To make the latter, the pP$_{CUP1}$-*myc-UBI* cassette contained in the smaller BamHI–ClaI fragment from pMRT7 (Rubio-Texeira and

Kaiser, 2006), was transferred to pRS423 digested with the same restriction sites. The pMRT39 construct was employed for coexpression of *myc*-Ubi and Gap1 mutant form Y395C (from YCpGAP1^{Y395C}) in the strain MRT507 (*gap1*Δ *ura3-52 his3*Δ) which was obtained by crossing between 10.560-4a and IH73. Strains MRT512 (*opt1*Δ *dal5*Δ *ptr2*Δ) and MRT513 (*opt1*Δ *dal5*Δ *ptr2*Δ *gap1*Δ) were also constructed in the Σ1278b background by PCR amplification of the corresponding *kanMX4* deleted ORFs from the corresponding BY deletion collection mutants and subsequent transformation and crossing of Σ1278b of opposite mating type. The sequences for all the oligonucleotides used for these deletions are described online in the *Saccharomyces* Genome Deletion Project web site (http://www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html). For construction of the mRFP-tagged strains the same wild-type Σ1278b strain 23.344c was transformed with the mRFP::KanMX6 cassette previously amplified by PCR from pFA6-mRFP::KanMX6 (Huh *et al*., 2003). To introduce the mutation K9R, K16R, an internal piece of *GAP1* ORF was deleted by replacement with *URA3* in the genome. A forward oligonucleotide containing the (–175)–(–135) bp region of *GAP1* plus homology to *URA3* cassette in pRS316 (5′-GAAGGTGAAGTCCACTTAAAT GAATGTCAATGAGACGATGAGATTGTACTGAGAGTGCAC -3′) and a reverse oligonucleotide containing the (+432)–(+394) of *GAP1* plus homology to *URA3* cassette in pRS316 (5′-ACTCACCCAGAGCCATAACCATAGCGTAAATCATGGT ACCCTGTGCGGTATTTCACACCG-3′) were used to amplify the replacement *URA3* fragment. The strain was subsequently transformed with the corresponding *GAP1* ORF piece amplified from YCpGap1^{K9R,K16R} plasmid (Soetens *et al*., 2001) using the forward oligonucleotide (5′-GATTTGGT AACTGATAAG-3′) and the reverse oligonucleotide (5′-CAACCAACCATTGTAACA-3′). Selection of the replacement took place in 5-FOA. For microscopy experiments the plasmids pGAP1-GFP or pGAP1^{Y395C}-GFP were transformed in either 21.983c or in the mRFP strains (genomic *GAP1*-mRFP, MRT287; genomic *gap1*^{K9R,K16R}-mRFP, MRT291).

All experiments were performed with nitrogen-starved cells, the cells were cultured at 30°C into exponential phase (OD_{600} = 1.5–2) in minimal medium, containing 0.17% (w/v) Difco yeast nitrogen base without amino acids and without or with 0.5% ammonium sulphate, and 2% glucose, supplemented with complete mixture without uracil or without uracil and histidine (CSM-Ura, or CSM-Ura-His, from MP Biomedicals). Exponential-phase cells were harvested, suspended in nitrogen starvation medium (NSM), containing 0.17% (w/v) Difco yeast nitrogen base without amino acids and without ammonium sulphate and 4% glucose, and incubated under shaking for 24 h at 30°C.

Growth assay

Nitrogen-starved glucose-repressed cells were diluted to an OD_{600} of 0.1 in fresh nitrogen starvation medium containing 4% glucose, supplemented with 5 mM of the indicated amino acid. Growth was measured via automated OD_{600} measurements using a BioscreenC apparatus (Labsystems). Serial 1/10 dilutions from an initial 0.5 OD_{600} ml^{-1} were spotted on 2% agar plates of the same medium but containing 1 instead of 5 mM of the indicated amino acid.

Biochemical determinations

Trehalase activity after addition of amino acids was determined in crude cell extracts as previously described (Donaton *et al*., 2003). Cells starved for nitrogen were collected for 30 min on ice, harvested, washed twice with Mes/KOH buffer (25 mM, pH 6) and resuspended in fresh nitrogen starvation medium with 4% glucose at a density of 25 mg wet weight per ml. The glucose liberated was assayed by the glucose oxidase/peroxidase method by adding 200 μl of GOD-PAP (Dialab). The protein level was determined by the Lowry procedure. The specific trehalase activity is expressed as nmol glucose liberated min^{-1} (mg protein)$^{-1}$.

Transport assays

Amino acid transport in intact cells was assayed by the use of [^{14}C]-labelled L-citrulline (Perkin Elmer), L-lysine (Perkin Elmer) and [^{3}H]-labelled L-histidine (ViTrax) as previously described (Donaton *et al*., 2003) as well as custom-made [^{14}C]-labelled L-Asp-γ-L-Phe (ViTrax). Transport activity is expressed as nmol substrate transported min^{-1} (mg protein)$^{-1}$.

For SCAM analysis, 10 mM (final concentration) 2-aminoethyl methanethiosulphonate, hydrobromide (MTSEA) (Toronto Research Chemicals) was added to *gap1*Δ cells expressing pFL38-Gap1, pFL38-Gap1^{S388C}, or pFL38-Gap1^{V389C}, 10 min before addition of amino acid. MTSEA was dissolved in nitrogen starvation medium just before use.

Fluorescence microscopy

For fluorescent localization studies, imaging was carried out with an Olympus FV1000 confocal laser scanning biological microscope, and images were processed with the accompanying software, FV10-ASW 2.0.

Protein extraction, immunoprecipitation and Western blot analysis

For detection of Gap1 and its oligo- and poly-ubiquitinated states, P13 fractions were isolated from cells expressing endogenous Gap1 or from a plasmid, GFP-tagged versions, based on the protocol described by Dupre and Haguenauer-Tsapis (2001). Prior to treatment nitrogen-starved cells were collected by centrifugation and resuspended in fresh nitrogen starvation medium supplemented with 10 μM $CuSO_4$ and pre-incubated for 30 min at 30°C for mild induction of myc-Ubi expression (full induction of *CUP1* promoter is usually achieved by 100 μM $CuSO_4$; Helliwell *et al*., 2001). After this pre-incubation cells were exposed to the nitrogen sources under study. Nitrogen-starved yeast cells (40 OD_{600} units) exposed for different times to the corresponding nitrogen compound were harvested by centrifugation and washed twice in distilled water plus 10 mM sodium azide. All subsequent steps were carried out at 4°C. Cell pellets were suspended in 200 μl of extraction buffer [0.1 M Tris-HCl (pH 7.5)-0.15 M NaCl-5 mM EDTA (pH 8.0), plus a mixture of protease inhibitors (Complete; Roche); 1 mM phenylmethylsulphonyl fluoride (PMSF) and 25 mM freshly prepared *N*-ethylmaleimide to prevent artefactual deubiquitination].

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Cells were broken with glass beads and the resulting homogenate was centrifuged at 3000 r.p.m. for 3 min to remove unbroken cells and debris. The supernatant was collected and centrifuged for 60 min at 13 000 *g*. The resulting (P13) pellet was suspended in 400 μl of extraction buffer plus 5 M urea, incubated at 0°C for 30 min, and centrifuged for 60 min at 13 000 *g*. The protein pellets were then suspended in 320 μl of extraction buffer plus 80 μl of 50% trichloroacetic acid. After incubation at 0°C for 30 min, the samples were centrifuged for 60 min at 13 000 *g*. The TCA protein precipitates were then neutralized in 25 μl of 1 M Tris base plus 25 μl of 2× sample buffer [100 mM Tris-HCl, pH 6.8, 4 mM EDTA, 4% sodium dodecyl sulphate (SDS), 20% glycerol, 0.002% bromophenol blue] containing 0.1 M DTT and heated at 37°C for 15 min. Aliquots of these plasma membrane-enriched fractions were analysed by Western blot as described below.

For Western blot detection of Gap1, purified monoclonal, horseradish peroxidase-(HRP)-conjugated anti-GFP rabbit antibody (Miltenyi Biotec), or primary polyclonal rabbit anti-Gap1 antibody (kindly provided by B. André, Brussels) were used. Gap1 primary antibody was detected with horseradish peroxidase-conjugated anti-rabbit antibodies (Amersham) (Rubio-Texeira *et al*., 2012). Normalization of the P13 fractions was achieved based on detection of Pma1 with goat polyclonal anti-Pma1 antibody (yN-20; Santa Cruz Biotechnology) detected in turn by HRP-coupled donkey anti-goat IgG, sc-2020 (Santa Cruz Biotechnology). Western Blot signals were developed using SuperSignal West Pico Chemiluminescent HRP substrate Kit (Thermo Scientific, Pierce). For imaging and quantification, ImageQuant Mini LAS4000 (GE Healthcare Life Sciences), Image Reader and Aida/1D Evaluation software were used. Luminescent Arbitrary Units (LAU) were assigned to each intensity peak corrected for background, as indicated by the software.

Reproducibility of the results

All experiments were repeated three to four times. Standard deviations for biological replicates of amino acid transport measurements are shown as error bars for comparisons between independent data points. Representative results are shown for comparisons between collections of interdependent data points (time-course measurements). The maximal extent of trehalase activation was variable between different experiments but the differences reported between controls and samples were consistently reproducible.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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